## Mobile genetic elements of nosocomial pathogens

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# SUMMARY AND GENERAL DISCUSSION

The research described in the first part of this thesis is focussed on the role and characteristics of mobile genetic elements. These DNA elements give bacteria the ability to pass on traits, such as antibiotic resistance mechanisms and virulence factors. Since bacteria are continuously exchanging DNA, a rapid emergence of multidrug resistant microorganisms occurs. Due to a lack of fallback agents, we are facing problems we thought we would never face again: the loss of a cure for bacterial infections! This first part addresses the different aspects of both the epidemiology and molecular characteristics of antibiotic resistance (Chapters 1, 2, 3A and 3B) and its relation with mobile DNA elements.

The second part of the thesis addresses the function of two highly variable genomic islands in *P. aeruginosa* [1], namely a family of filamentous bacteriophages that has been hypothesized to play a role in biofilm formation and pyocins (Chapter 4 and 5).

## PART 1. Mobile genetic elements and antibiotic resistance

The world is facing a global pandemic of antibiotic resistance which must be tackled soon or as experts say, we will return to the pre-antibiotic era [2]. This will not only affect treatment of infectious diseases, but will also lead to a roll-back of major milestones in modern medicine, such as major surgery, organ transplantation and cancer treatment [2]. The most important disease threat in Europe is already thought to come from antibiotic resistant micro-organisms, and without a global response on the use and abuse of antibiotics, this threat will only increase [3]. Therefore, understanding the epidemiology and molecular aspects of antibiotic resistance is of critical importance to address methods to control the ongoing spread of antibiotic resistance.

The nature of ciprofloxacin resistance in a collection of *E. coli* isolates was studied in Chapter 1 of this thesis. Ciprofloxacin, a fluoroquinolone, functions by inhibiting the bacterial type II topoisomerase/DNA gyrase [4]. Point mutations in the genes for type II topoisomerase and DNA gyrase (*parC*, *parE*, *gyrA*, and *gyrB*) have been long considered as the primary cause of ciprofloxacin resistance. In *Escherichia coli*, alterations in the GyrA subunit are possible because the sequence of this gene is by nature highly variable [5]. Anti-microbial pressure selects for a subset of these natural mutations, which easily results in a fluoroquinolone-resistant population. However, recently it has been shown that besides these intrinsic resistance mechanisms, ciprofloxacin resistant populations are also associated with the presence of specific DNA elements [6-8]; [9]. Since the rapid emergence of antibiotic resistant subpopulations is often caused by horizontal gene transfer of resistance genes, it is not surprising that a novel plasmid-mediated quinolone resistance mechanism was identified [10]. The genes responsible for quinolone

resistance, *qnr* and AAC(6')-lb-cr, are mainly found within integron class 1 structures, but could also be found in transposon-like elements (*qnrS*).

Since we observed a significant increase in the frequency of ciprofloxacin-resistant *E. coli* strains in the haematology departments of two university hospitals in The Netherlands, we analysed the possible mechanisms of this resistance. We were able to show that the increase was not due to the emergence of unique ciprofloxacin-resistant clones. Eighty-one % of the ciprofloxacin-resistant isolates contained an *intI1* gene, compared to 11% of the ciprofloxacin-susceptible isolates ($p < 0.0001$). We could not detect the quinolone resistance gene *qnr*A in any of the integrons nor in dot-blot hybridisation of total DNA. Finally, conjugation experiments showed that ciprofloxacin resistance was not co-transferred with class 1 integrons. We did show that the ciprofloxacin-resistant isolates harboured mutations in the *gyrA* gene, which are known to encode ciprofloxacin resistance. These mutations might be ascribed to the ciprofloxacin pressure as part of the selective decontamination of the digestive tract of the patients included in this study. It is known that ciprofloxacin pressure leads to DNA damage and the induction of SOS response. A connection between ciprofloxacin induced SOS response and mobile DNA transfer has been made in *Vibrio cholerae* and *Staphylococcus aureus* [11, 12] A 100 kilobase integrative conjugating element, harbouring multiple antibiotic resistance genes, was transferred upon induction with ciprofloxacin in *Vibrio cholera* [11], and in *S. aureus* ciprofloxacin induction lead to horizontal dissemination of pathogenicity island-encoded virulence factors [12]. These observations lead to the possibility that the selective ciprofloxacin pressure results in increased/induction of horizontal gene transfer, which in turn lead to the acquisition of class 1 integrons. In conclusion, an association was observed between ciprofloxacin resistance and the presence of class 1 integrons, which could not be explained by the genetic determinants of quinolone resistance known at the time of the study.

Since the discovery of plasmid-mediated quinolone resistance, only one class of antibiotics remains that is not affected by these resistance mechanisms. This class of antimicrobial agents is that of the polypeptides, and primarily comprises the cationic antimicrobial peptides polymyxin B and polymyxin E (colistin). The use of these antibiotics was partially abandoned in the 1960s because of concerns about their toxicity [13]. Recently, reassessment of the safety of polymyxin B and colistin showed that both antibiotics can be safely and adequately used to treat multidrug-resistant infections, especially respiratory tract infections. Unfortunately, the first polymyxin-resistant clinical isolates have already been isolated [14]. Although the resistance mechanism is not fully understood, it is thought to occur through adaptive intrinsic resistance, rather than through plasmid-mediated resistance. Polymyxins target the bacterial LPS and through lipid-A modification of LPS bacteria prevent access of the drug to the bacterial cell wall and subsequent killing. Although Gram-negative bacteria have several lipid-A modification enzymes, to our knowledge genes encoding for lipid-A modification enzymes have not yet been found on mobile DNA elements, such as plasmids. Since polymyxins are often the last relevant therapeutic option for treatment of infections caused by multidrug resistant Gram-negative bacteria, they should be used cautiously to prevent development of polymyxin resistance.

In Chapter 2, the epidemiology and molecular aspects of carbapenem resistance in *Acinetobacter baumannii* in a university hospital in Turkey were studied. The study started with the observation of a rapid increase in the isolation of antibiotic resistant microorganisms, especially carbapenem-resistant *A. baumannii* strains, isolated from patients admitted to the intensive care unit (ICU). In contrast to the study described in Chapter 1, genomic typing revealed three predominant genotypes. The largest clonal outbreak consisted of 72 clinical strains, isolated from 60 different patients. Molecular characterization showed the presence of the oxacillinase genes, $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-51-like}}$ in the predominant genotypes. The gene $bla_{\text{OXA-58}}$ is consistently associated with carbapenem resistance, whereas OXA-51-like enzymes are also widespread in carbapenem-susceptible strains [15] [16]. Insertion sequences, such as IS*aba*1, provide promoters that enhance gene expression when adjacent to $bla_{\text{OXA-51-like}}$, and as such may contribute to the carbapenem resistance [17]. The presence of insertion sequences adjacent to $bla_{\text{oxa-51-like}}$ needs to be further evaluated to determine the contribution of $bla_{\text{oxa-51-like}}$ genes to the carbapenem resistance in these clinical isolates. In 2006, Vahaboglu *et al*. already reported that $bla_{\text{oxa-58}}$-bearing plasmids were quickly spreading among multiple clones of the $bla_{\text{oxa-51-like}}$ *A. baumannii* strains in Turkey [18]. This, in combination with extensive use of carbapenems and a lack of strict infection control measures, most likely led to this large outbreak. As discussed earlier in the introduction, one of the key features that facilitate the emergence of *A. baumannii* is its ability to survive in dry conditions over prolonged periods. An investigation was started and an environmental isolate related to the major outbreak clone was recovered from a ventilator monitor, thus confirming the ability of *A. baumannii* to survive in the hospital environment. Implementation of strict infection control measures have led to control the outbreak, but did not eradicate carbapenem-resistant strains from the ICU yet. This might partially be caused by the difficulties in completely removing this pathogen from the hospital environment. Irrespective of the antibiotic pattern, *Acinetobacter* species need more stringent conditions than typically sufficient in order to eradicate all bacteria [19]. Seventy percent of ethanol is often used for decontamination purposes, but is however ineffective against *Acinetobacter baumannii* [20]. This shows the importance of adapting disinfection procedures to the bacterial pathogen encountered in the hospital environment, in order to fully eradicate the microorganism from the environment.

The careful use of antibiotics, surveillance proGrammes, and strict infection control measures are thought to be responsible for the relatively low resistance levels observed in The Netherlands. In Chapter 3A of this thesis we evaluate clonal dissemination after the implementation of the new guidelines for highly resistant micro-organisms as published by the Dutch Working Party on Infection Control [21] [22] [23]. The incidence density of highly resistant micro-organisms (HRMO) observed was considered very low, 4.3 per 10.000 patient days [21]. Due to a lack of uniform definitions of highly resistant microorganisms, comparisons of our data with those from other studies are difficult. HRMOs identified in this study showed that clonal dissemination occurred in 4 occasions with Gram-negative rods, and in 4 occasions with Gram-positive cocci [21]. Unsurprisingly, clonal dissemination was primarily associated with patients staying on the intensive care unit. On this unit contact between nursing staff and these critically ill patients is very intense, which increases the risk for cross-transmission. Moreover, these patients are often immune-

compromised and therefore treated with antibiotics to prevent opportunistic infections. As a consequence, only antibiotic resistant bacteria survive, thereby increasing the resistance rate.

A subsequent study on the contribution of horizontal gene transfer on antibiotic resistance follows in Chapter 3B. Integron class 1, an antibiotic resistance gene capture and expression system, was identified in nearly 70% of the highly resistant Gram-negative micro-organisms identified in the study presented in Chapter 3A. Characterization of gene cassettes of these integrons revealed 22 unique and 2 prominent types. Subsequent epidemiological analysis showed that resistance genes in bacterial isolates obtained from seven patients (out of a total of 57) were acquired via horizontal gene transfer, most likely of integron harbouring plasmids. As in the two previous studies, dissemination was shown to occur especially in patients admitted to intensive care units. In Chapter 3A we have shown that clonal dissemination occurred in 0.7 / 10.000 patients, in this complementary study horizontal gene transfer of antibiotic resistance genes, occurred almost twice as often. This shows the importance of horizontal gene transfer in the dissemination of antibiotic resistance genes between patients in a hospital environment.

As discussed in the introduction, bacterial resistance represents a form of evolution in which bacteria try to circumvent the effects of antibiotics. Subsequent epidemiological analysis showed that resistance genes in bacterial isolates obtained from seven patients (out of 57) were acquired via horizontal gene transfer. Thus far, bacteria have been able to develop resistance mechanisms against all known classes of antibiotics. It is therefore reasonable to assume that bacteria will eventually develop new resistance mechanisms against any antimicrobial agent, whether it belongs to old classes or to novel ones. Therefore, compounds that do not aim to eradicate, but rather inhibit virulence factors might be less prone to the development of bacterial resistance mechanisms. Since many bacteria use quorum sensing systems to coordinate virulence, this might be a promising target. For example, two quorum sensing inhibitors, garlic extract and 4-nitro-pyridine-*N*-oxide (4-NPO) were found to reduce *P. aeruginosa* biofilm formation and infection in a *Caenorhabditis elegans* pathogenesis model [24]. By knocking out bacterial virulence factors only, the bacteria undergo less pressure, which potentially results in less evolutionary pressure to develop resistance mechanisms. At the moment, several studies focus on the identification of novel bioactive compounds directed against bacterial virulence factors, such as type III secretion systems. A second advantage of these novel anti-virulence compounds is that they will more specifically target pathogenic bacteria and leave most of the normal host micro flora unharmed. This specific treatment could also help to avoid secondary infections due to a microbial misbalance and therefore a further reduction of the use of antibiotics.

## PART 2. Variable genetic elements of *Pseudomonas aeruginosa*

Besides antibiotic resistance, bacteria also posses other variable genetic islands, often involved in virulence. The last two Chapters of this thesis describe two of these elements of the opportunistic pathogen, *Pseudomonas aeruginosa*.

In Chapter 4 we describe the characterization of filamentous bacteriophages of *P. aeruginosa* belonging to the Pf family [25]. These filamentous bacteriophages were hypothesized to be involved in the formation of small colony variants and biofilm formation [26]. Since biofilm formation is a major virulence trait of *P. aeruginosa* we studied these bacteriophages in more detail. Our study showed that the integrated filamentous bacteriophage of *P. aeruginosa* strain PA14 (Pf5) is not involved in small colony formation, and therefore probably not in biofilm formation. We also tested 48 clinical isolates and found no association between the ability to form small colony variants and the presence of the Pf filamentous bacteriophages. Population analysis of *P. aeruginosa* clinical isolates has shown that the most frequent genotype is represented by strain PA14, whereas strain PAO1 was found to be a rare genotype [27]. Together this shows that the pf-like filamentous bacteriophages are generally not involved in small colony formation of *P. aeruginosa*.

This study does however show that filamentous phages of the Pf type are present in a large number of *P. aeruginosa* strains, as was also shown in other studies. Moreover, these phages generally seem to be active, since phage replication forms can be isolated and the chromosomal insertion sites differ considerably between different strains. This could mean that the Pf phages do provide a specific advantage for *P. aeruginosa* strains, although at present it is not known what kind of advantage, since the small colony phenotype is not dependent on these phages. At the very least Pf phages play, because of their active replication and variable genome locations, a role in *P. aeruginosa* strain differentiation and evolution. It may however also provide a competitive advantage in e.g. the lungs, as it has been shown for other genomic prophages [28]. The impact of this evolution on the development of virulence remains to be investigated, but should not be underestimated. Bacteriophages have been shown previously to play an important role in the evolution of various pathogens, such as the emergence of *Salmonella enterica* serovar *Typhi* [29]. Perhaps in the future we will witness that also *P. aeruginosa* will develop a specific lineage that is no longer a general pathogen but a specific human pathogen.

In Chapter 5 of this thesis, we studied gene regulation of pyocins. Pyocins represent, together with the Pf phages, probably the most variable genetic islands of clinical *P. aeruginosa* isolates [1], although their role in *P. aeruginosa* evolution and survival in the host is unclear. In this chapter we describe a novel cell surface signalling system that regulates the expression of pyocins [30]. This surface signalling system contains an iron starvation ECF sigma factor and as such is likely to be involved in the uptake of heterologous iron. In microbial populations, bacteria have to compete with each other for their nutrients, such as iron. Therefore, they produce iron-scavenging siderophores. Some bacteria, like *P. aeruginosa*, are able to use also heterologous siderophores, produced by other bacteria. This is the first report that

bacteriocins are produced upon the presence of a specific extracellular signal indicating the presence of other bacteria. The presence of heterologous siderophores indicates the presence of other bacterial species nearby and is then used to trigger the production of antimicrobial pyocins. In this way, Pseudomonas can combine two different competition mechanisms. Identification of the heterologous siderophore could prove this hypothesis, which would pinpoint to an unexpected complexity in bacterial behaviour, whereby bacteria are anticipating the presence of bacterial competitors.

## General Conclusions

Antibiotic changed the world for the better, but without a global response against the rising antibiotic resistance levels, the world will change for the worse. Mobile genetic elements confer bacteria with unique ways to exchange genetic information. As such, mobile DNA elements are an important cause of the rapid spread of antibiotic resistance and provide bacteria opportunities to acquire novel virulence factors.

Other variable genetic elements, such as phages are studied in this thesis. Although at the moment the consequences of the presence of these variable DNA elements for therapy are perhaps minor compared to the presence of antibiotic resistance genes, gene rearrangements and gene acquisition of toxin-encoding genes on this filamentous bacteriophage could have major consequences in the future.

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